*NATURAL IMMUNITY TO BACTERIAL INFECTIONS: THE RELATION OF COMPLEMENT TO HEAT-LABILE OPSONINS**

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Communicated June 2, 1969

Abstract.—Heat-labile opsonins to pneumococci in normal mammalian sera, unlike antibodies, fail to interact with the bacteria at 0°C and require Ca^{++} and/or Mg^{++}. They are readily removed from serum by antigen-antibody complexes that fix complement (C) and are inhibited by reagents that inactivate various C components. The principal heat-labile opsonin to pneumococci is activated C3 ($C3_b$), but a slight enhancing effect is exerted by one or more of the late-reacting components of the hemolytic complement system (C5–C9). Since heat-labile opsonins are immunologically polyspecific, they presumably play a broad protective role in the early (preantibody) phase of acute bacterial infections.

The nature of the heat-labile factors in normal mammalian sera that promote phagocytosis of many pathogenic bacteria has long been a matter of controversy.[1, 2] Recently, Hirsch and Strauss[1, 3] have concluded that heat-labile opsonins are neither natural antibodies nor the complement system. Their exclusion of complement was based primarily on a failure to demonstrate a requirement for divalent cations.[4] Li and Mudd,[5] on the other hand, have reported that ethylenediaminetetraacetic acid (EDTA) blocks the action of heat-labile opsonins on *Staphylococcus aureus*. The general confusion that exists on the relation of complement to phagocytosis has recently been reviewed.[2]

The present studies on the heat-labile opsonins to *Diplococcus pneumoniae* (pneumococcus) were undertaken in an effort to define the role of complement in antibacterial phagocytic defense. Pneumococcus was selected as the test organism because its pathogenicity is relatively well understood.[6] Its virulence depends primarily on the antiphagocytic properties of its capsule; once ingested by a phagocytic cell, it is promptly destroyed.

Two mechanisms of phagocytosis are known to participate in the destruction of pneumococci in the infected host: (a) *surface phagocytosis*, which operates in the preantibody phase of pneumococcal disease and may occur in the absence of opsonins of any kind; and (b) *immune phagocytosis*, which occurs later in the disease when enough anticapsular antibody has accumulated to opsonize the encapsulated organisms.[6] Surface phagocytosis is quantitatively less efficient than immune phagocytosis.

The experiments summarized in this report define a third mechanism that involves the participation of heat-labile opsonins. Since these opsonins are immunologically polyspecific and are present in normal serum, they presumably play a significant role in early antipneumococcal defense.

Methods.—All phagocytic tests were done with leucocytes from acute peritoneal exudates harvested 20 hr after a single injection of starch-aleuronat suspension.[7] The volumes

injected in rats, mice, and guinea pigs were 5.0 ml, 1.5 ml, and 15 ml, respectively. Aliquots containing 2.5×10^8 cells washed in Hanks' bovine albumin–glucose solution (HBG)[8] were centrifuged in the cold at 180 *g* for 5 min in 13 × 100-mm screw-cap tubes, and the supernatant in each tube was decanted.

In phagocytic tests done with type 25 pneumococci (Pn25) supplied by Dr. Robert Austrian, Department of Research Medicine, University of Pennsylvania, 1 ml of the desired test medium and 0.1 ml of a washed bacterial suspension containing 1.25×10^{10} log phase organisms per ml were added to the packed cells. The ratio of bacteria to cells was thus 5:1. After being thoroughly mixed and tumbled end over end at 12 rpm for 30 min at 37°C, the cells in each tube were diluted in 2 ml of HBG, centrifuged at 180 *g* for 5 min (to remove most of the extracellular bacteria), and examined microscopically in smears stained with methylene blue. The percent phagocytosis was determined by counting the percentage of 400 granulocytes containing one or more bacteria.

In tests involving the type 1 pneumococcus (Pn1),[7] the volume of test medium added was reduced to 0.1 ml, and the ratio of bacteria to cells was increased to 8:1. These changes were made to increase the incidence of particle-cell collisions and thus partially compensate for the greater virulence of the type 1 organism, which is not phagocytized in the more dilute system.[9]

Each comparative phagocytic experiment was done repeatedly to determine the reproducibility of the results. Only representative values of reproducible experiments are recorded in the tables.

Results.—The relation of heat-labile opsonins to complement: As shown in Table 1, heat-labile opsonins in normal rat serum have a profound effect on the phagocytosis of both type 25 and type 1 pneumococci *in vitro*. Complement fixation by added antigen-antibody complexes removes these opsonins from

TABLE 1. *Comparative properties of heat-labile opsonins to type 25 (Pn25) and to type 1 pneumococci (Pn1) in normal rat serum.*

	Per cent phagocytosis	
	Pn2 5	Pn1
HBG*	0	3
Normal rat serum (NRS)	90	35
Heated NRS (56°C for 5 min)	0	2
Zymosan-treated serum (25.0 mg/ml)	2	5
NH_4OH-treated serum (30 mM), diluted 1/5	8	—†
EDTA-treated serum (6.0 mM)	1	1
EDTA-treated serum (6.0 mM) + $CaCl_2$ (6.0 mM)	93	26
Venom-treated serum (5 μg/ml)	0	0

Each of the procedures used to inactivate complement components in the serum reduced the CH_{50} titer to less than 5% of its original value.

* HBG = Hanks' bovine albumin–glucose solution.[8]

† Not done.

normal serum,[10] as does treatment with zymosan, NH_4OH, EDTA, and cobra venom[4] (Table 1). Furthermore, Ca^{++} (and/or Mg^{++}) is required for opsonization of the bacteria (Table 2), as well as for removal of the heat-labile opsonins by adsorption.[10] Collectively, these results, which have been duplicated with mouse, rabbit, and guinea pig sera, suggest the involvement of C1, C4, C2, and C3. The inactivation of heat-labile opsonins by cobra venom, which destroys C3–C9 without significantly affecting the C1, C4, and C2,[12, 19] indicates that C3 and/or one or more of the later acting components (C5–C9) play a critical role in the opsonization process.

TABLE 2. *Requirement of divalent cations for opsonization of Pn25 by pretreatment with heat-labile opsonins in normal rat serum.*

Opsonizing medium	Per cent phagocytosis
Normal rat serum (NRS)	63
NRS + EDTA (6.0 mM)	1
NRS + EDTA (6.0 mM) + $CaCl_2$ (12.0 mM)	66

The organisms were incubated in the opsonizing medium for 30 min at 37°C and were then washed once in balanced salt solution (BSS),[11] suspended in BSS, and added to the phagocytic system (see *Methods*). The serum was undiluted except for the reagents added, which never exceeded 0.1 ml per ml. The pH of the undiluted serum was not significantly lowered by the acid formed from the EDTA–$CaCl_2$ reaction.

The role of C3: When pneumococci were incubated in normal guinea pig serum, they consumed a larger percentage of C3 than any of the other eight complement components (Fig. 1). Since C3 is the most plentiful of the complement components in guinea pig serum,[14] it is clear that the principal component consumed in the reaction was C3.

Monospecific anti-C3 serum from rabbits immunized with purified guinea pig C3[15] caused type 25 pneumococci, pretreated with normal guinea pig serum, to agglutinate. In addition, it caused their capsules to swell (quellung reaction). Similarly, goat anti-rabbit globulin labeled with fluorescein isothiocyanate[16] stained specifically the capsules of pneumococci that had been pretreated with normal guinea pig serum and the monospecific anti-C3 rabbit serum.

When 6.25×10^8 encapsulated type 25 pneumococci were twice incubated at 37°C for 30 minutes in 1 ml of normal guinea pig serum containing 10 μg of purified ^{125}I-labeled C3,[17] the organisms, after being washed in HBG, still retained 5.3 per cent of the total radioactivity of the serum. In contrast, the

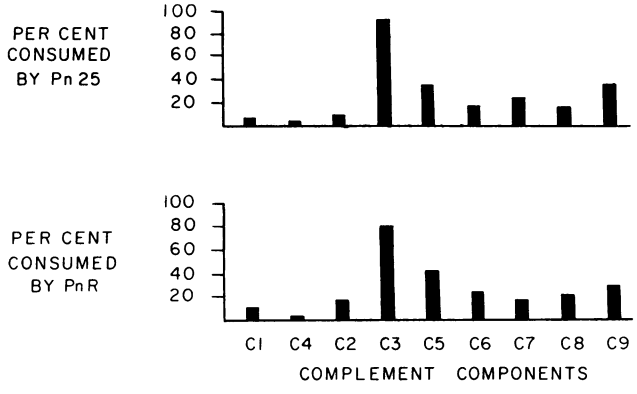

FIG. 1.—Percentage of complement components in pooled normal guinea pig serum consumed by interaction with encapsulated (*Pn25*) and unencapsulated (*PnR*) pneumococci. The organisms were harvested in the log phase, washed twice, and resuspended in Hanks' bovine albumin–glucose solution (HBG)[8] at a concentration of 1.25×10^{10} per ml. 0.05 ml of the suspension was added to 1 ml of the serum and incubated for 30 min at 37°C. After removal of the organisms by centrifugation, the serum was titrated for the residual complement component.[13] Their titers are expressed as percentages of the respective components contained in normal serum that had been similarly incubated with 0.05 ml of HBG as a control.

uptake from heated serum[18] was 1.3 per cent and from serum containing 0.01 *M* EDTA only 0.9 per cent.

Addition of 0.86 μg of a purified fraction[19] of cobra venom to 1 ml of guinea pig serum destroyed more than 95 per cent of the C3 activity of the serum without causing a detectable loss of C1, C4, or C2. Whereas phagocytosis of encapsulated pneumococci was virtually nil in the venom-treated serum, the opsonic activity was fully restored by the addition of purified C3 (Table 3).

TABLE 3. *Ability of purified C3 to restore the opsonizing activity to normal guinea pig serum pretreated with a purified factor from cobra venom.*

Suspending medium	Per cent phagocytosis	
Normal guinea pig serum (NGS)	83	85
Cobra-venom-treated NGS	0	<1
Cobra-venom-treated NGS + C3	82	85
C3 in HBG	<1	0
HBG	0	0

To inactivate C3–C9, 0.86 μg of venom[19] was added to 1 ml of NGS and incubated at 37°C for 1 hr. To restore C3 activity in the venom-treated serum, 1.2 mg of purified C3[15] in 0.08 ml of 0.05 *M* sodium chloride and 0.005 *M* pH 5 phosphate buffer was added to 1 ml of the serum. When the C3 was tested alone as an opsonin, the same amount was added to 1 ml of HBG. The phagocytic tests were done with encapsulated pneumococci (Pn25) and with guinea pig exudate leucocytes. The results of two representative experiments are recorded.

All these findings indicate that C3 becomes fixed in the capsule of the organism where it participates in the opsonization process. The failure of C3 alone to opsonize the organisms (Table 3, line 4) suggests that it must be converted, presumably to $C3_b$, before it can function as an opsonin.

The role of later-acting components: Since cobra venom may inactivate C5–C9, as well as C3, the question arises as to how much of a role the late-acting components of the complement system play in the opsonization process. To answer this question, studies were performed with C5-deficient mouse serum.[20, 21] Its promotion of phagocytosis, as shown in Table 4, was slightly less than that of coisogenic normal mouse serum,[21] and the deficiency was at least partially reversible with purified C5.[22] This finding was corroborated by the demonstration that the C5-deficient mice are slightly less resistant to a carefully calibrated pneumococcal challenge than are their coisogenic relatives (Fig. 2).

Thus, it is evident that heat-labile opsonins to pneumococcus consist primarily of C3 but are slightly enhanced by one or more of the late-acting components of the hemolytic complement sequence.

TABLE 4. *Phagocytosis of encapsulated pneumococci (Pn25) in sera of coisogenic C5-deficient and C-normal mice.*

	Per Cent Phagocytosis				
Suspending medium	Expt.: 1	2	3	4	5
C-normal serum	35	36	35	51	33
C5-deficient serum	22	26	25	34	19
C5-deficient serum + purified guinea pig C5	27	39	33	41	26

The phagocytic tests were done with both isologous and homologous exudate leucocytes. The purified guinea pig C5[22] used to restore the activity of the C5-deficient mouse serum was added in a concentration of 15 μg per ml in the first four experiments and 150 μg per ml in the fifth.

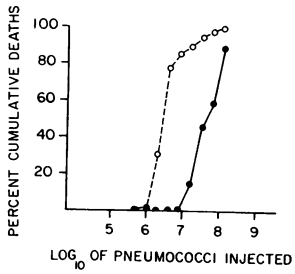

FIG. 2.—Comparative dose–response curves of C-normal (●—●) and C5-deficient (O---O) mice to intraperitoneal injections of varying doses of an intermediate capsular variant of type 3 pneumococcus (Pn3-int).[23] Ten mice were inoculated at each dose level. The LD_{50}, calculated by the Reed-Muench method, was $10^{6.6}$ for the C5-deficient mice and $10^{7.6}$ for the C-normal mice.

Discussion.—It seems reasonable to assume that fixation of the necessary C factors on the bacteria is mediated by antibody and the usual sequential activation of complement components. The natural antibodies involved, however, must be to cell-wall antigens, since an excess of homologous capsular polysaccharide does not interfere with phagocytosis promoted by heat-labile opsonins.[10] The putative cell-wall antigens are presumably shared by many bacterial species, i.e., are immunologically polyspecific.

In related studies of erythrophagocytosis, Nelson found that sensitized sheep erythrocytes carrying antibody and EAC1, 4, 2*a*, 3*b* are readily phagocytized, whereas cells carrying antibody and only EAC1, 4, 2*a* are not.[24]

The results of the present experiments are of particular interest in relation to the recent detection of C3 reactive sites on the surfaces of monocytes[25, 26] and granulocytes.[25] It is at these sites that the heat-labile opsonins in normal mammalian sera probably function as immunologically polyspecific ligands.

The authors are grateful to M. M. Mayer for much helpful advice, to A. G. Osler and B. Hill for some of the complement determinations, and to J. Messenheimer, C. Kohstall, and K. Laudenslayer for excellent technical assistance.

* These studies were supported from 1964 to 1966 by the Commission on Streptococcal and Staphylococcal Diseases of the Armed Forces Epidemiological Board (contract DA 49-193 MD 2313). Since August 1966 they have been supported by the National Science Foundation (grant GB 5387) and also, during the past year, by the National Science Foundation (grant GB 2597), the Office of Naval Research (contract 248-60), and the U.S. Public Health Service (grants AI-02566 and 5 TI-AI 282).

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